ボンは弱いエストロゲン受容体結合能を有するものの、タモキシフェン等の抗エストロゲン剤が持つエストロゲン受容体阻害作用はほとんど認められない。以上の結果から、大豆イソフラボンの乳癌抑制効果としてエストロゲン受容体を介さない作用機作も考慮に入れるべきと考える。当該研究で樹立した各細胞株を駆使することで、大豆イソフラボンによる乳癌抑制効果がエストロゲン受容体依存的か否か、さらにその本態が細胞増殖抑制にあるのか、あるいは細胞死誘導にあるのか明らかになろう。また得られた結果は、内分泌かく乱作用を有さない乳癌予防物質や薬剤の発見、創製へ向けた基盤となるものと期待される。

E. 結論

ヒト正常型遺伝子トランスジェニックラット1個体に発生した乳腺腫瘍より乳癌細胞株7株（C1、C2、C3、C6、C11、C15、C17）を樹立した。ER 遺伝子発現の解析では、ER 陰性1株、ERαのみ陽性1株、ERβのみ陽性2株、ERα，β陽性3株であった。C3（ERα，β陽性）、C11（ERβ陽性）、C17（ER陰性）細胞の増殖はいずれもエストロゲン非依存的であった。C3細胞とC11細胞に対して 50μM ゲニステインには有意な DNA 合成抑制を認めたが、ダイゼインは無効であった。C11 細胞を用い 10μM ゲニステイン添加後の遺伝子発現変動を DNA マイクロアレイにより解析すると、添加 72 時間後に2倍以上の発現変動を認めた遺伝子は、発現上昇 410 個、低下 327 個の 737 遺伝子で、全体の 3.6%（737/20,500）であった。発現上昇群には eaf2、IL-15、tumor necrosis factor receptor superfamily 12、caspase recruitment domain protein 9、発現低下群には transcription factor 2a1、connexin 26、apoptosis inhibitor 2、cyclinG が含まれており、ゲニステインがエストロゲン非依存的乳癌細胞の転写活性抑制ならびにアポトーシス誘導により乳癌抑制作用を発揮している可能性を示唆している。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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2) Hamaguchi, T., Matsuoka, Y., Kawaguchi, H., Fukamachi, K., Takasuka, N., Ueda, S., Shimizu, K., Ohki, M., Kusunoki, M., Sakakura, T., Yoshida H., and Tsuda, H. Terminal endbuds and acini as the respective major targets for chemical and sporadic carcinogenesis in the mammary glands of human c-Ha-ras protooncogene transgenic rats. *Breast Cancer Res. Treat.*, 83: 43-56, 2004.

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2. 学会発表

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H. 知的所有権の取得状況

なし

図1

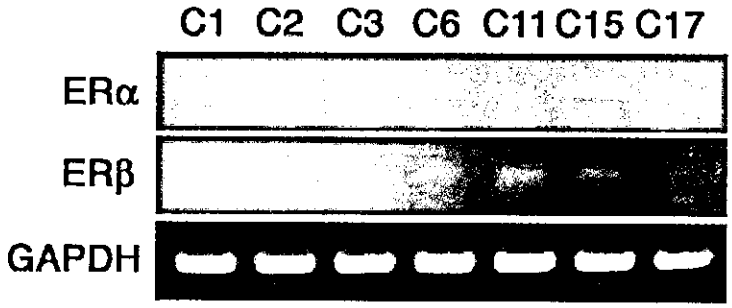
C1 C2 C3 C6 C11 C15 C17
ERα
ERβ
GAPDH

図2

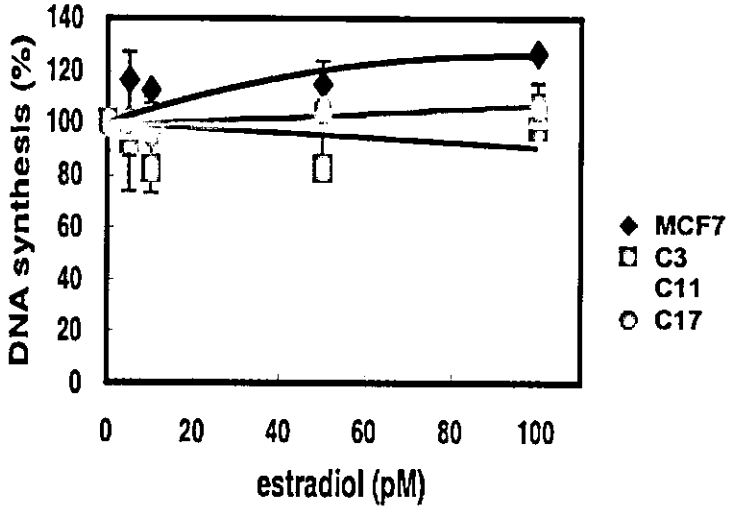
DNA synthesis (%)
140
120
100
80
60
40
20
0
0 20 40 60 80 100
estradiol (pM)
MCF7
C3
C11
C17

図3

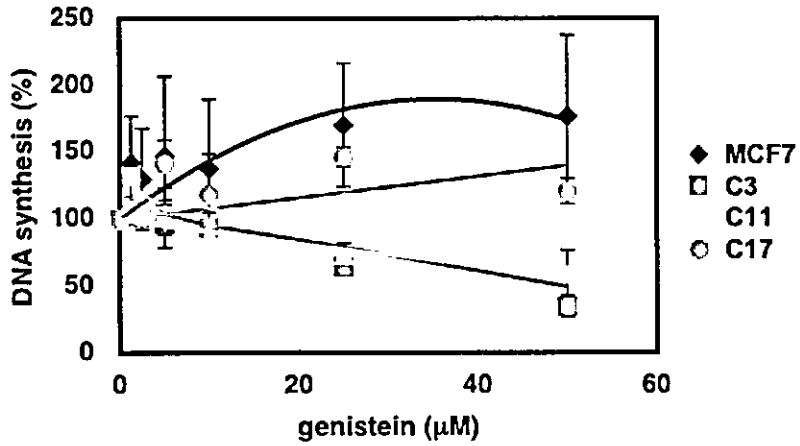
250
200
150
100
50
0
DNA synthesis (%)
0
20
40
60
genistein (μM)
MCF7
C3
C11
C17

図4

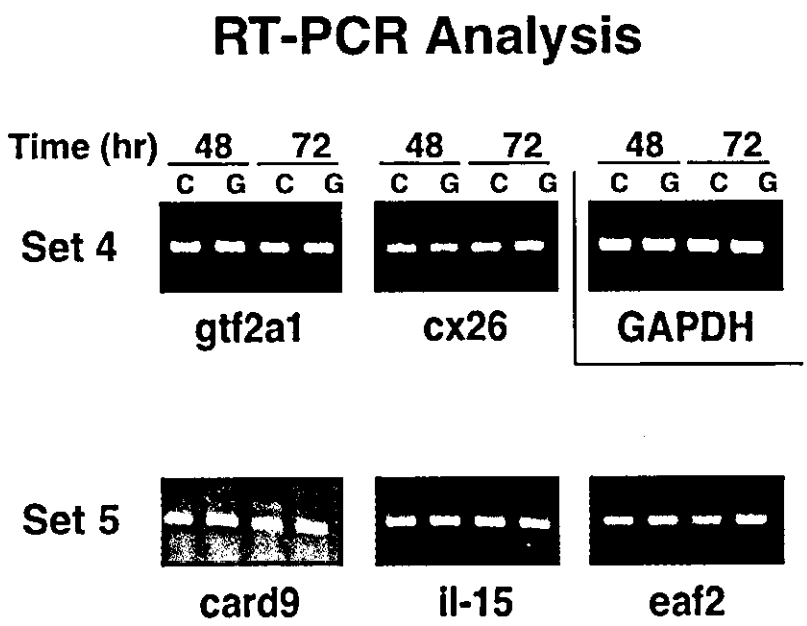
RT-PCR Analysis
Time (hr)
48
72
48
72
48
72
C G C G C G C G C G C G
Set 4
gtf2a1
cx26
GAPDH
Set 5
card9
il-15
eaf2

表1

Cell line	Sequence of gene				
	p53	Rat *Ha-ras*[a]		Human *Ha-ras*[b]	
		exon 1	exon 2	codon 12	codon 61
C1	W[c]	W	W	GA/TC	W
C2	W	W	W	GA/TC	CGG
C3	W	W	W	GAC	W
C6	W	NA[e]	NA[e]	GA/TC	W
C11	GGC(codon 246[d])	W	W	GAC	CAT
C15	W	W	W	GA/TC	CAT
C17	W	W	W	GAC	W

[a]The entire region flanking exons 1 and 2 including codons 12 and 61, respectively, were sequenced.
[b]The wild-type sequences are as follows: codon 12 is GGC and codon 61 is CAG.
[c]W denotes the wild-type sequence.
[d]The wild-type sequence of codon 246 is CGC.
[e]The rat *Ha-ras* was not sequenced because of unsuccessful PCR amplification.

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takatori S, Kitagawa Y, Oda H, Miwa G, Nishikawa J, Nishihara T, Nakazawa H, Hori S.	Estrogenicity of Metabolites of Benzophenone Derivatives Examinaed by a Yeast Two-Hybrid Assay.	J Health Sci	49	91-98	2003
Nikaido Y, Yoshizawa K, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N, Tsubura A.	Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring.	Reprod Toxicol	18	803-811	2004
Yuri T, Nikaido Y, Shimano N, Uehara N, Shikata N, Tsubura A.	Effects of prepubertal zeranol exposure on estrogen target organs and *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis in female Sprague-Dawley rats.	In Vivo	18	755-762	2004
Hamaguchi T, Matsuoka Y, Bechberger J, Ohnishi T, Fujita K, Naus CC, Kusunoki M, Tsubura A, Tsuda H.	Establishment of an apoptosis-sensitive rat mammary carcinoma cell line with a mutation in the DNA-binding region of p53.	Cancer Lett			in press
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Nikaido Y, Danbara N, Tsujita-Kyutoku M, Yuri T, Uehara N, Tsubura A.	Effects of prepubertal exposure to xenoestrogen on development of the estrogen target organs in female CD-1 mice.	In Vivo			in press

Estrogenicity of Metabolites of Benzophenone Derivatives Examined by a Yeast Two-Hybrid Assay

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(Received September 19, 2002; Accepted December 24, 2002)

The estrogenic activities of S-9 metabolites of benzophenone derivatives (benzophenone, 2-hydroxy-4-methoxybenzophenone, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-octyloxybenzophenone, 2,4-dihydroxybenzophenone and 2,3,4-trihydroxybenzophenone) and benzhydrol were examined with a yeast two-hybrid screening system. After chemicals were incubated in an S-9 mix at 37°C for 4 hr prior to their incubation with the yeast strain, the S-9 mix containing metabolites was assayed for the estrogenic activity by the yeast two-hybrid assay. Benzophenone, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone exhibited estrogenic activities after incubation with the S-9 mix. The estrogenic metabolites of 2-hydroxy-4-methoxybenzophenone were fractionated by high-performance liquid chromatography, one of which was identified as 2,4-dihydroxybenzophenone. This assay will be a useful tool for detecting proestrogens.

Key words —— yeast two-hybrid assay, benzophenone, estrogenic metabolite

INTRODUCTION

Benzophenone is listed among the "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the U.S.A., and the Ministry of Environment in Japan. However, benzophenone is an important compound in everyday life because of its ability to absorb and dissipate ultra violet (UV) light.[1] Its twelve derivatives, designated as benzophenone-1 through benzophenone-12, are used in cosmetics and sunscreens to protect human skin and hair from UV irradiation. 2-Hydroxy-4-methoxybenzophenone (benzophenone-3, BZ-3) is one of the most widely used UV absorbers for sunscreens on the market. Orally or topically administered BZ-3 is converted to at least three metabolites, 2,4-dihydroxybenzophenone (benzophenone-1, BZ-1), 2,3,4-trihydroxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone (benzophenone-8, BZ-8).[2–5] BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities in an *in vitro* assay system using MCF-7 cells.[6] Benzophenone is converted to an estrogenic metabolite, *p*-hydroxybenzophenone.[7] Thus, benzophenone derivatives can be categorized as proestrogens. However, the estrogenic activities of the metabolites of benzophenone derivatives have not been fully elucidated.

We have developed a novel assay procedure for detecting the hormonal activities of chemicals using a yeast two-hybrid system.[8] We tested the estrogenic activities of various chemicals, and found that a phenol with a hydrophobic moiety at the para-position is the key structural moeity of estrogenic chemicals.[9] The phenyl or phenylether residues of lipophilic chemicals can be converted to a phenol residue by drug metabolizing enzymes. These facts imply that some chemicals exert their estrogenic activities by metabolic activation *in vivo*. The endocrine activities of pesticides and natural products can be affected by metabolism.[10,11] For example, methoxychlor (MXC) is metabolized to 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane to exert estrogenic activity.[12] The EDSTAC final report recommends that the evaluation of chemicals using *in*

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vitro high throughput prescreens should be performed in the presence and absence of metabolically active extracts to detect proestrogens.[13] There are few reports of assay procedures that are able to evaluate the estrogenic activity of metabolites.[14–16] In mutagenicity testing, incubation with an S-9 extract mixture has been the standard method for *in vitro* metabolic activation.[17] Here we apply a yeast two-hybrid assay for detection of estrogenic activity after metabolic activation by incubation with an S-9 extract mix (S-9 mix), and examine the estrogenic activities of metabolites of benzophenone derivatives.

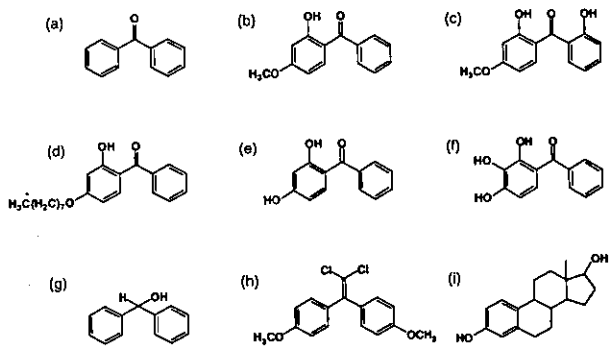

Fig. 1. The Structure of Chemicals Examined in This Assay

a), benzophenone; b), BZ-3; c), BZ-8; d), 2-hydroxy-4-octyloxybenzophenone (BZ-12); e), 2,4-dihydroxybenzophenone (BZ-1); f), 2,3,4-trihydroxybenzophenone; g), benzhydrol; h), MXC, i), E_2.

MATERIALS AND METHODS

Chemicals —— 17-β-Estradiol (E_2, > 97.0%), MXC (> 97.0%), benzophenone (> 98.0%), 2,4-dihydroxybenzophenone (> 98.0%), 2,3,4-trihydroxybenzophenone (> 98.0%), and benzhydrol (> 98.0%) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Hydroxy-4-methoxybenzophenone (> 98.0%), 2,2′-dihydroxy-4-methoxybenzophenone (> 98.0%) and 2-hydroxy-4-octyloxybenzophenone (> 98.0%) were purchased from Aldrich Chem. Co. (Milwaulkee, WI, U.S.A.). All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

Activation by an S-9 Fraction —— S-9 extracts (rat liver 9000 × *g* supernatant fraction induced with phenobarbital and 5,6-benzoflavone) and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). To a tube containing 990 μl of the S-9 mix (S-9 mix: 20 μl S-9, 0.8 μmol NADPH, 0.8 μmol NADH, 1.0 μmol glucose-6-phosphate, 0.4 U G6PDH, 20 μmol Na_2HPO_4, 20 μmol NaH_2PO_4, 6.6 μmol KCl and 1.6 μmol $MgCl_2$) 10 μl of each test chemical dissolved in dimethyl sulfoxide (DMSO) was added and then incubated at 37°C for 4 hr. The chemicals, after incubation with the S-9 mix, were stored at –80°C until their application to the yeast two-hybrid strain. The heat-inactivated S-9 extract was prepared by incubation at 95°C for 5 min, and used for the negative control experiments. The structures of chemicals examined in this paper are shown in Fig. 1.

Yeast Two-Hybrid Assay for Detecting Estrogenic Activity after Metabolic Activation —— In this study, we used the yeast two-hybrid system with the estrogen receptor, estrogen recepter α (ERα), and the coactivator, transcriptional intermediary factor 2 (TIF2), as previously described.[8,9] Yeast cells carrying the pGBT9-estrogen receptor ligand binding domain (pGBT9-ERLBD) and pGAD424-TIF2 plasmids were grown overnight at 30°C with vigorous shaking in selective medium (S.D. medium lacking tryptophan and leucine). The yeast cells, resuspended in 2 × S.D. medium made up at twice the usual concentration, were mixed at a 1 : 1 (v/v) ratio with the test chemicals, which had been treated with the S-9 mix, and then incubated at 30°C for 4 hr. Aliquots of cells were withdrawn and washed by centrifugation. The cell density was determined by measurement of the absorbance at 595 nm. A lysate was prepared by enzymatic digestion of the cells with 1 mg/ml Zymolyase 20T at 37°C for 15 min. The lysate (200 μl) was mixed with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (40 μl) and incubated at 30°C for 30 min. The enzymatic reaction was stopped by the addition of 1 M Na_2CO_3 (100 μl). β-Galactosidase activity was calculated as described previously.[8] Estrogenic activity was also tested by MCF-7 proliferation assay[18] and a reporter gene assay using HeLa cells.[19]

HPLC Analysis of BZ-3 Metabolites —— BZ-3 (1.0×10^{-4} M) was incubated with the S-9 mix as mentioned above. The metabolites after S-9 activation (1.5 ml) were extracted twice with 3.0 ml ethylacetate. The extracts were dried under an N_2 stream and dissolved in 30 μl methanol. The extracts (15 μl) were applied to a reverse phase HPLC column (Cadenza CD-C18, 4.6 × 250 mm, 3 μm; Imtakt, Kyoto, Japan). LC-10AD pumps were used with a DGU-14A degassing unit and C-R7A integrator (Shimadzu, Kyoto, Japan). The HPLC column was eluted with a 75% methanol/water at a flow rate of

1.0 ml/min. The eluate was monitored at 230 nm with an SPD-10AV detector (Shimadzu). Each 0.5 ml fraction was collected and dried under an N_2 stream, and dissolved in 10 μl DMSO to be applied to the yeast two-hybrid strain for testing estrogenic activity.

Liquid Chromatography/Mass Spectrometry Analysis of BZ-3 Metabolites —— Liquid chromatography/mass spectrometry (LC/MS) analysis was performed on an API3000 (Applied Biosystems, Foster City, CA, U.S.A.) equipped with an electrospray ionization (ESI) interface and an Agilent 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler and a G1379A degasser. The column used was a reverse phase HPLC column (Cadenza CD-C18, 2.0 × 100 mm, 3 μm; Imtakt). The mobile phases consisted of 100% acetonitrile (A) and 1% aqueous acetic acid (B). Elution was performed using a linear gradient from 30% A to 80% A during 30 min at 0.2 ml/min. The ESI interface was control by Analyst software (v.1.2). ESI-MS was operated in negative or positive ion mode. The heated capillary and voltage were maintained at 500°C with and –/+4.2 kV (negative/positive mode), respectively. Mass spectra were measured from *m/z* 50 up to *m/z* 300.

RESULTS

Estrogenic Activity of Metabolites of Benzophenone Derivatives

The negative control experiments were performed using an S-9 mix containing inactive S-9 extracts (an inactive S-9 mix). Serial dilutions of E_2 were incubated with the inactive S-9 mix at 37°C for 4 hr, and then the reaction mixtures were applied to the yeast two-hybrid assay. Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2a). The concentration of E_2 showing 10% of the 1.0×10^{-7} M activity (relative effective concentration, REC_{10}) was 1.7×10^{-10} M. Under these conditions BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities. Their REC_{10} values were 6.5×10^{-7} and 6.8×10^{-6} M, respectively. Benzophenone, BZ-3, BZ-8, BZ-12, benzhydrol and MXC did not exhibit estrogenic activities.

The metabolic activation experiments were performed using an S-9 mix containing active S-9 extracts (an active S-9 mix). Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2b). The REC_{10} of E_2 was 2.5×10^{-10} M. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. Their REC_{10} values were 2.5×10^{-5}, 1.5×10^{-4}, 1.4×10^{-5} and 1.0×10^{-5} M, respectively. The estrogenicities of BZ-1 and 2,3,4-trihydroxybenzophenone were reduced by incubation with the active S-9 mix. Their REC_{10} values were 1.5×10^{-4} and 2.1×10^{-5} M, respectively.

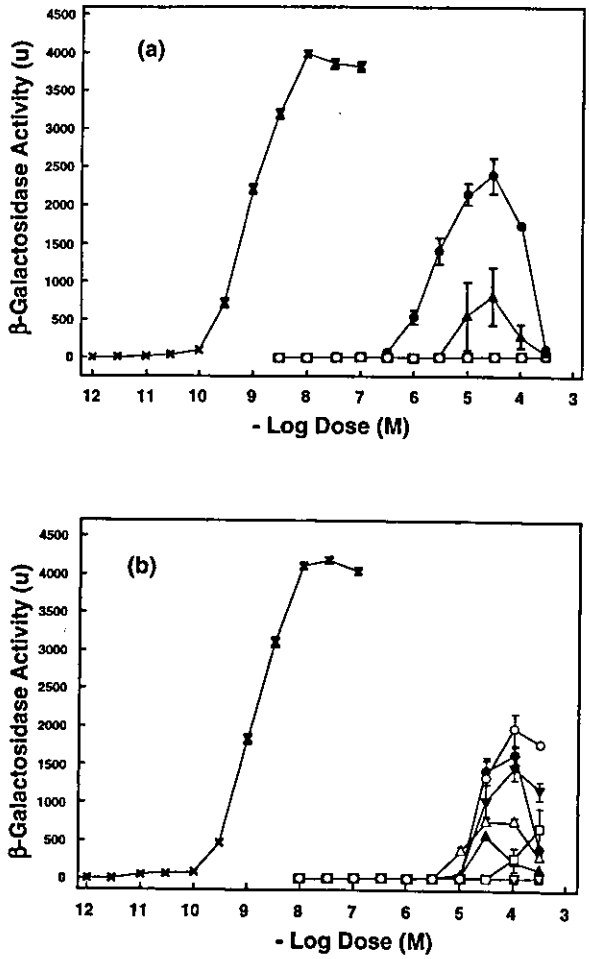

Fig. 2. Dose–Response Curve for Chemicals Incubated in Inactive S-9 Mix (a) and in Active S-9 Mix (b)

Values are means of three separate experiments (bars: S.D.). Benzophenone, (□); BZ-3, (○); BZ-8, (△); BZ-12, (▽); BZ-1,(●); 2,3,4-trihydroxybenzophenone, (▲); benzhydrol, (■); MXC, (▼); E2, (×).

HPLC Analysis of the Metabolites of BZ-3

After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix, the metabolites were extracted with ethylacetate and then fractionated by HPLC. Three

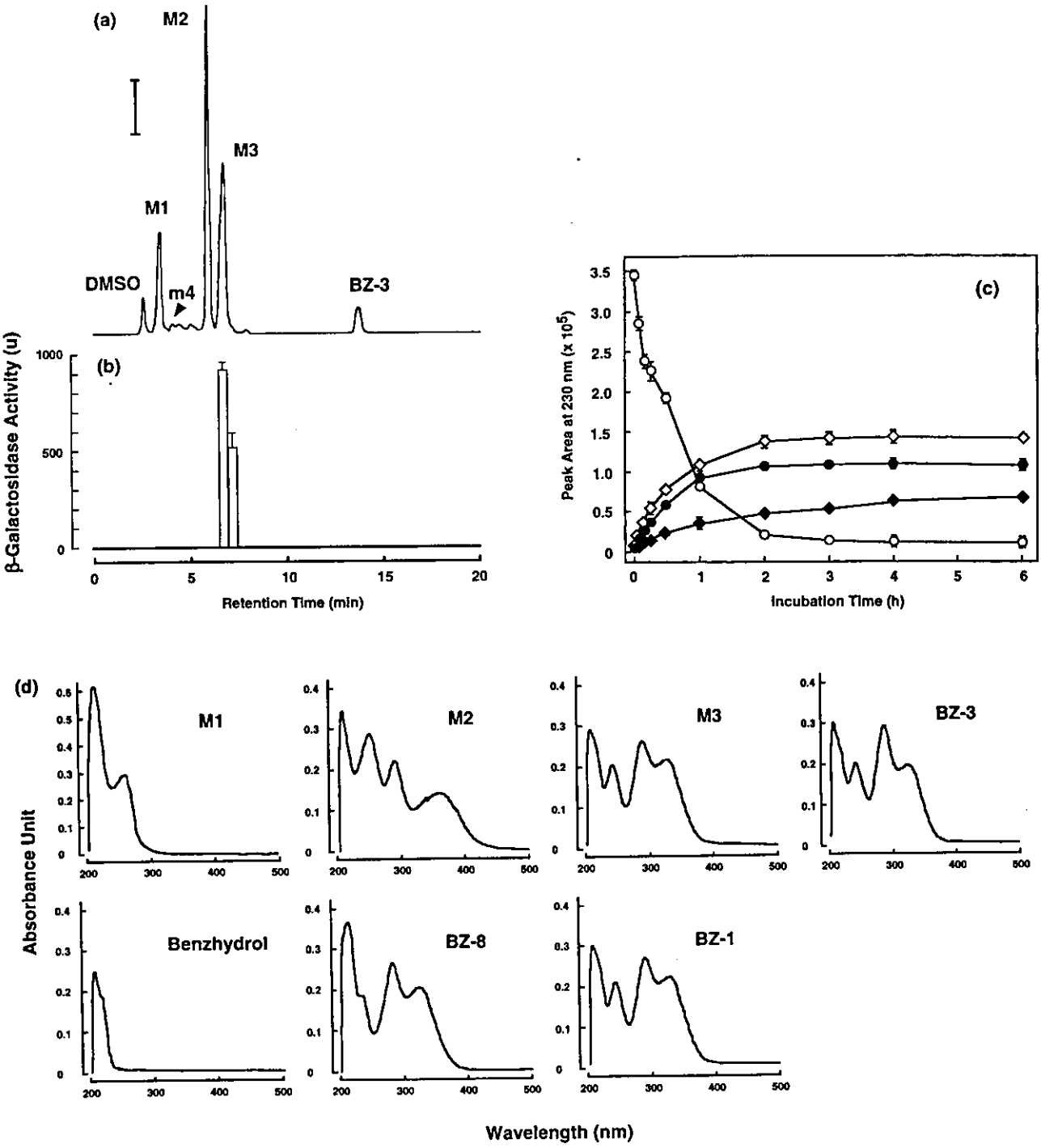

Fig. 3. The HPLC Analysis of the Metabolites of BZ-3 (a)

Bar expresses 0.2 auf. at 230 nm. The retention times were as follows (min): DMSO, 2.6; M1, 3.5; m4 (2,3,4-trihydroxybenzophenone), 4.1; M2, 5.9; M3 (BZ-1), 6.8; BZ-8, 8.1; BZ-3, 13.8. The estrogenic activities of the fractions (b). Values are means of three separate experiments (bars: S.D.). Changes in the levels of BZ-3 and its major metabolites, M1, M2 and M3 (c). Points express the peak area of recorded at 230 nm. Values are means of three separate experiments (bars: S.D.). BZ-3, (○); M1, (◆); M2, (◇); M3,(●). The concentration of BZ-3 at zero time was $9.5 \pm 0.1 \times 10^{-5}$ M. The UV spectra of M1, M2, M3, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol (d).

major metabolites (M1, M2 and M3) were detected (Fig. 3a). The retention time of a minor metabolite (m4) corresponded to that of 2,3,4-trihydroxybenzophenone. Each fraction was also examined for its estrogenic activity using the yeast assay system. The fractions containing M3 exhibited estrogenic activities (Fig. 3b). M1, M2 and M3 produced from BZ-3 concurrently (Fig. 3c). UV spectra of metabo-

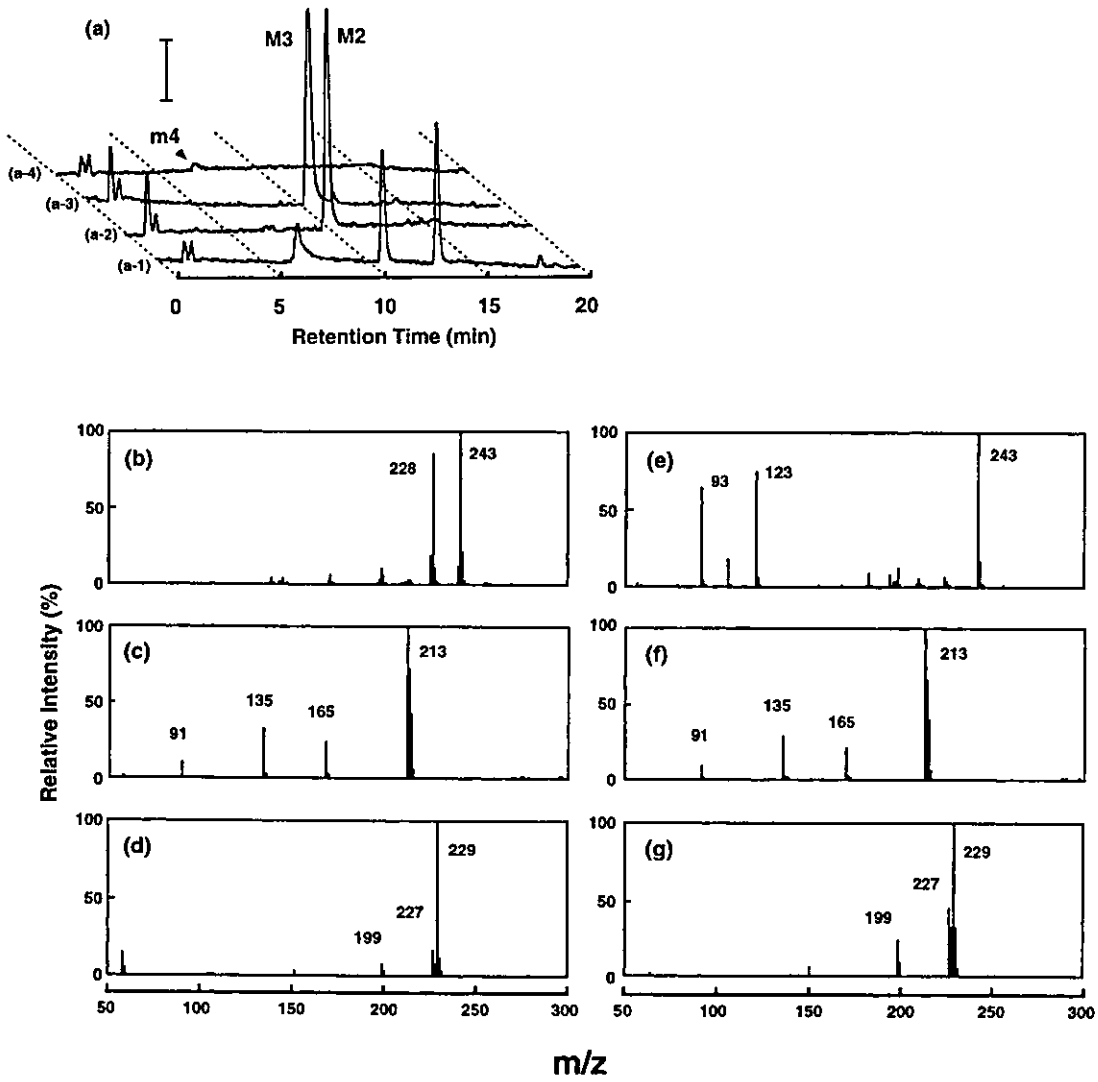

Fig. 4. Total Ion Chromatograms of BZ-3, BZ-8, BZ-1, 2,3,4-Trihydroxybenzophenone, M2, M3 and m4 (a)

Mixture of benzophenone derivatives containing 2.0×10^{-5} M BZ-3, BZ-8, BZ-1, and 2,3,4-trihydroxybenzophenone (a-1), M2 (a-2), M3 (a-3), m4 (a-4). Bar expresses the intensity of 1.0×10^{8} cps. The retention times were as follows (min): m4 (2,3,4-trihydroxybenzophenone), 6.7; M2, 10.0; M3 (BZ-1), 10.9; BZ-8, 13.5; BZ-3, 18.5. The mass spectra of M2 (b), M3 (c), m4 (d), BZ-8 (e), BZ-1 (f) and 2,3,4-trihydroxybenzophenone (g).

lites, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol were exhibited in Fig. 3d. Both benzhydrol and M1 did not have absorption above 280 nm. M2 absorbed from 200 to 440 nm with maxima at 290 and 360 nm and minima at 277 and 326 nm. M3 absorbed from 200 to 400 nm with maxima at 289 and 325 nm and minima at 262 and 312 nm. The profiles of UV spectrum and retention time in HPLC analysis of M3 were identical to those of BZ-1. After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix for 4 hr, the concentrations of M3 (BZ-1) and BZ-3 were $3.3 \pm 0.1 \times 10^{-5}$ and $2.0 \pm 0.2 \times 10^{-6}$ M, respectively. To obtain further information about the metabolites, LC/MS analysis was also performed. The total ion chromatograms (negative ion mode) of M2, M3 and m4 were exhibited Fig. 4a. The mass spectra of M2, M3, m4, BZ-1 and 2,3,4-trihydroxybenzophenone were shown in Figs. 4b–4g, respectively. The base peak was detected at *m/z* 243 in the mass spectrum of M2. The mass spectra of M3 and m4 were identical to those of BZ-1 and 2,3,4-trihydroxybenzophenone, respectively. M1 did not detectable in both negative and positive ion mode.

DISCUSSION

P450 enzymes in the active S-9 mix are able to convert E_2 to its oxidative metabolites.[20] The dose–

response curves of E_2 after the incubation with the active S-9 mix were almost identical to that after the incubation with the inactive S-9 mix containing medium (Figs. 2a and b). Under these conditions, degradation of E_2 in the active S-9 mix containing medium was not observed (data not shown). The active S-9 mix fraction is able to mix with yeast cells suspended in 2 × S.D. medium without the need for additional measures.

The hydroxyl group of E_2 at the 3-position plays an important role in activating the ER.[21] Without the S-9 mix activation, benzophenone derivatives with a hydroxyl group at the 4-position, BZ-1 and 2,3,4-trihydroxybenzophenone, exhibited estrogenic activities. Some compounds containing phenol residues, such as *p*-alkylphenols, parabens and bisphenol A, exhibit estrogenic activities.[9,22–24] The phenol residues of these compounds are believed to participate in mimicking E_2 at the ER ligand-binding domain.[9] The phenol residue in benzophenone derivatives would also play such a role in activating ER. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. MXC is designated as a proestrogen for its conversion to an estrogenic metabolite, 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane.[12] It was also confirmed by us that benzophenone after metabolic activation showed estrogenic activity as well in the proliferation assay and the reporter gene assay using cultured cells (data not shown). This assay system is applicable for the detection of the proestrogens. We demonstrated that BZ-3 was converted to an estrogenic metabolite BZ-1 by incubation with the active S-9 mix. BZ-8 has an additional hydroxyl group at 2′-position of BZ-3. The estrogenic activities of its metabolites would depend on 2,2′,4-trihydroxybenzophenone and/or metabolites with hydroxyl group at a 4′-position. With both incubation with the inactive and the active S-9 mix, BZ-12 exhibited no estrogenic activity. The bulky octyloxy group moiety may prevent hydroxylation and/or ER activation.

BZ-3 is metabolized to BZ-1, BZ-8 and 2,3,4-trihydroxybenzophenone *in vivo*.[2–5] In the assay system, BZ-3 was metabolized to M1, M2, M3 (BZ-1) and m4 (2,3,4-trihydroxybenzophenone). The structures of M1 and M2, non-estrogenic metabolites, were inferred by examination of their UV spectra and LC/MS analysis. M1 was assumed to be a benzhydrol derivative for its lack of absorption above 280 nm. Benzhydrol could be one of the major metabolites of benzophenone in hepatocytes,[2–5] and had no absorption above 250 nm in methanol. Instead of formation of BZ-8, an unknown metabolite, M2, was detected. Compared to BZ-3, the UV spectrum of M2 was shifted to a longer wavelength. The mass spectrum suggested a molecular weight (M.W.) for M2 of 244. The M.W. of BZ-3 is 228.2. The UV and mass spectra of M2 were quite different from those of BZ-8 (Figs. 3d, 4b and 4e). M2 would be formed by the hydroxylation in the aromatic ring with methoxy and hydroxyl groups of BZ-3.

BZ-3 is used in many cosmetics and sunscreens as a UV-absorber.[1] The compound can be absorbed topically and converted to an estrogenic metabolite, BZ-1.[25,26] We demonstrated that BZ-3 was converted to the estrogenic metabolite, BZ-1, in a 33% yield by incubation with S-9 mix for 4 hr. At the same condition, 2,3,4-trihydroxybenzophenone was produced in a less than 1% yield (data not shown). Thus, the yield of non-estrogenic metabolites including M1 and M2 based on BZ-3 was approximately 60%. From 1 to 10% of BZ-3 in cosmetic products penetrates human skin.[25,26] These facts suggest that BZ-1 is produced *in vivo* by those applying a sunscreen or a cosmetic containing BZ-3. UV absorbers are increasingly used as a result of growing concern about UV irradiation and skin cancer. Schlumpf, *et al.* reported that other UV absorbers, such as 4-methyl-benzylidene camphor and octyl-methoxycinnamate, also exhibit estrogenic activity.[27] Studies of the effects on endocrine systems by UV absorbers should be performed more extensively, because of their use in children.

This assay system was able to detect the conversion of BZ-3 to an estrogenic metabolite in a minimum number of steps. This yeast two-hybrid system is able to evaluate the effects of chemicals on thyroid hormone receptors and androgen receptors by changing pairs of the receptors and coactivators to the relevant pairs.[8] Studies of the thyromimetic and anti-thyromimetic activities of metabolites of chemicals are ongoing in our laboratory. This assay system will be a useful tool for the detection of prohormonal activities of chemicals.

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Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring

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Abstract

The objective of this study was to examine the effects of maternal exposure to xenoestrogen, at levels comparable to or greater than human exposure, on development of the reproductive tract and mammary glands in female CD-1 mouse offspring. Effects of genistein (GEN), resveratrol (RES), zearalenone (ZEA), bisphenol A (BPA) and diethylstilbestrol (DES) were examined. Beginning on gestational day 15, pregnant CD-1 mice were administered four daily subcutaneous injections with 0.5 or 10 mg/kg/day of GEN, RES, ZEA or BPA, 0.5 or 10 μg/kg/day of DES dissolved in dimethylsulfoxide (DMSO), or DMSO vehicle ($n = 6$). Vaginal opening was monitored, 6 animals per group were autopsied at 4, 8, 12 and 16 weeks of age and estrous cyclicity was monitored from 9 to 11 weeks of age. Maternal exposure to xenoestrogen accelerated puberty onset (vaginal opening) and increased the length of the estrous cycle; mice treated with GEN, RES, BPA or DES spent more time in diestrus, and ZEA-treated mice spent more time in estrus. Lack of corpora lutea and vaginal cornification were observed at 4 weeks of age in the high-dose GEN (33%) and RES (17%) groups, and in the high- and low-dose BPA groups (33 and 50%, respectively) and DES groups (83 and 100%, respectively). Lack of corpora lutea and vaginal cornification was observed in the high-dose ZEA group at 4, 8, 12 and 16 weeks of age (83, 100, 83 and 33%, respectively). Mammary gland differentiation was accelerated in ZEA- and BPA-treated mice with corpora lutea at 4 weeks of age. ZEA-treated mice without corpora lutea showed mammary growth arrest at 8, 12 and 16 weeks of age; their mammary glands consisted only of a dilatated duct filled with secreted fluid. Mammary gland growth was similar with xenoestrogens other than ZEA or BPA to that of the controls at all time points. High-dose GEN and RES and high- and low-dose BPA and DES exerted transient effects on the reproductive tract and mammary glands, whereas ZEA exerted prolonged effects.

Keywords: Genistein; Resveratrol; Zearalenone; Bisphenol A; Diethylstilbestrol; CD-1 mouse; Prenatal; Maternal

1. Introduction

Endocrine-disrupting chemicals such as xenoestrogens are naturally occurring substances (i.e., phytoestrogens and mycoestrogens) or synthetic chemicals that are released into the environment and can interfere with the endocrine system and exert various effects in vertebrates [1]. These effects can be severe, particularly in prepubertal children where endogenous estrogen concentration is low [2]. In utero exposure to diethylstilbestrol (DES; (*E*)-3,4-bis(4-hydroxyphenyl)-3-hexene) as an antiabortive induces clear cell adenocarcinoma of the vagina in daughters after puberty [3]. Although the risk among the DES exposed daughters for the development of clear cell adenocarcinoma is small (<1%), DES is linked to more frequent benign reproductive tract dysfunction and structural abnormality. Development of estrogen target tissues appears to be particularly vulnerable to effects of xenoestrogens during the prenatal period [4]. Mice are especially sensitive to estrogens in utero; exposure to natural or synthetic estrogens in utero can produce various postnatal effects [5].

Naturally occurring and synthetic chemicals that exhibit estrogenic activity are widely distributed in the environment [1]. Among the chemicals that exhibit such activities are genistein (GEN), resveratrol (RES), zearalenone (ZEA) and bisphenol A (BPA). GEN (4′,5,7-trihydroxyisoflavone) is a major component in soy-based foods and reports indicate that the level of GEN exposure in Asian populations consuming a soy-rich diet ranges from ~1 to 30 mg/day (~0.02–0.55 mg/kg/day) [6]. Infants consuming a diet of soy-based formula may be exposed to ~20–40 mg/day

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(4–6 mg/kg/day) of soy isoflavones, of which GEN accounts for >65% [7]. RES (*trans*-3,4′,5-trihydroxystilbene) is found in grapes and red wine [8]. Red wine is believed to be the main source of RES, and a person drinking one glass of red wine a day consumes ~0.02 mg/kg/day of RES [9]. ZEA (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resocylic acid-lactone) is a mycotoxin synthesized by *Fusarium* mold, and is present as a natural contaminant in food as a result of infection of grain by *Fusarium* species. Human exposure to ZEA in the United States is 1–5 mg/day (0.02–0.1 mg/kg/day) [10]. BPA (4,4′-isopropylidenediphenol), an industrial chemical that exhibits estrogenic action, is a monomer used in the manufacture of many chemical products including the interior lining of food and beverage cans, dental sealants and polycarbonate plastic products including baby bottles. Typical human exposure to environmental BPA ranges from 0.025 to 0.25 mg/kg/day [11]. To date, the estrogenic potency of these chemicals has not been compared.

The present study has compared the effects of early exposure to xenoestrogens in pregnant mice treated subcutaneously (s.c.) with each xenoestrogen at 0.5 mg/kg/day to mimic human-relevant doses, and also treated with a 20-fold higher dose (10 mg/kg/day). DES was included as a positive estrogenic control at doses approximately 1000 times lower than those of the other xenoestrogens (0.5 μm/kg/day or 10 μg/kg/day). Xenoestrogens interact with the estrogen receptor (ER) and evoke estrogenic activity [5]. The present dose of DES was based on the finding that DES has a 1000-fold higher affinity for ERα than GEN [12]. We examined the effects of these xenoestrogens on the reproductive tract and mammary glands.

2. Materials and methods

2.1. Test chemicals

GEN was purchased from Fujicco (Kobe, Japan), and RES, ZEA, BPA and DES were obtained from Sigma (St. Louis, MO). The purity of all tested chemicals was ≥99%. All chemicals arrived in powder form and were kept at 0 °C in the dark. Immediately before use, each chemical was dissolved in dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto), and stored at 4 °C.

2.2. Animals

Outbred Crj:CD-1 (ICR) timed pregnant mice were purchased from Charles River Japan (Atsugi), and arrived in our laboratory on day 14 of gestation.

2.3. Experimental environment

The animals were kept at 22 ± 2 °C and 60 ± 10% humidity, with a 12 h/12 h light/dark cycle. To avoid exposure to endocrine-disrupting chemicals, mice were housed in standard mouse polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River, Yokohama) as bedding. To avoid exposure to dietary phytoestrogens, mice were fed a low-phytoestrogen diet (NIH-07 PLD; Oriental Yeast, Chiba, Japan); NIH standard dietary pellets (NIH-07 open formula) contain phytoestrogens from soy products and alfalfa [13]. Water was supplied in polycarbonate bottles with rubber stoppers. Thus, exposure to known environmental endocrine-disrupting agents were minimized.

2.4. Experimental procedures

Beginning on gestational day 15, mice were given four daily s.c. injections of 0.5 or 10 mg/kg/day of GEN, RES, ZEA or BPA, 0.5 or 10 μg/kg/day of DES, or vehicle DMSO alone (untreated control). Doses were adjusted daily according to body weight, to provide constant dose levels.

Female offspring were weaned at 21 days of age. Timing of vaginal opening was recorded. In 12 mice from each group, vaginal smears were taken from 9 to 11 weeks of age, and estrous cycle was monitored. Six randomly selected mice in each group at 4, 8, 12 and 16 weeks of age were weighed, anesthetized, euthanized by cervical dislocation, and autopsied. Ovaries, uterus, vagina and inguinal mammary glands were fixed in 10% neutral buffered formalin. Mid-uterine transverse segments, vaginal transverse segments, the center of each ovary, and inguinal mammary glands were sectioned (thickness, 4 μm) and stained with hematoxylin and eosin (HE). Thoracic mammary glands were processed for whole-mount preparation and the degree of differentiation was arbitrarily scored from 1 to 4 using the following criteria: Score 1, low degree of differentiation, terminal end buds (TEBs) in the periphery with lateral buds but no alveolar development; Score 2, small number of alveoli in poorly developed ductal tree; Score 3, intermediate development of ductal and alveolar structure; Score 4, high degree of development, and lobulo-alveolar formation in the gland. Our experimental protocol was approved by the Animal Experimentation Committee, Kansai Medical University.

2.5. Statistical analysis

All data were expressed as mean ± S.E. After assurance of homogeneity of variance, analysis was performed using non-repeated measure ANOVA parametric test or Kruskal–Wallis non-parametric test. If the P value of these pre-tests was <0.05, post hoc analysis was performed using Fisher's protected least significant difference test. Differences between groups were considered significant if the P value was <0.05.

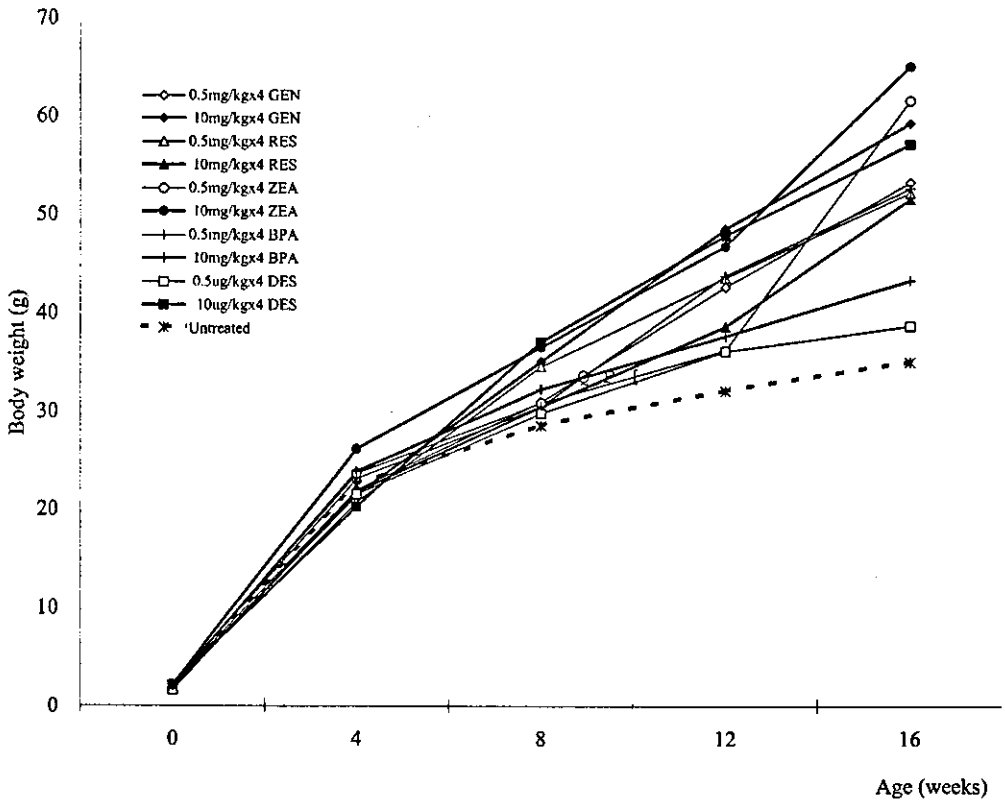

Fig. 1. Body weight gain in female CD-1 offspring of mothers administered four daily low- or high-dose injections of xenoestrogen, beginning on gestational day 15.

3. Results

3.1. Body weight gain in female offspring

Maternal exposure to test chemicals accelerated body weight gain in female offspring compared with untreated controls (Fig. 1). At 16 weeks of age, all mice other than the low-dose DES group were significantly heavier ($P < 0.01$, respectively) than untreated controls.

3.2. Vaginal opening

Xenoestrogens accelerated timing of vaginal opening, compared with untreated controls (Table 1). In the GEN, ZEA, DES and high-dose BPA groups, vaginal opening was significantly earlier. In the RES and low-dose BPA groups, there was no significant difference.

3.3. Estrous cycle

All untreated control mice exhibited a regular cycle of 5.2 ± 0.1 days, with 18.7 and 24.2% of time spent in estrus and diestrus phase, respectively (Table 2). Although all xenoestrogen-treated mice were cycling, cycle length was significantly elongated in all groups other than low-dose DES. In the GEN, RES, BPA and DES groups, the percentage of time spent in the diestrus phase was significantly longer than in untreated controls. In the ZEA groups, the percentage of time spent in the estrus phase was significantly longer than in untreated controls.

Table 1
Age at vaginal opening in female offspring of mothers exposed to xenoestrogen (four daily injections, beginning on gestational day 15)

Test chemical	Dose	Vaginal opening (days)
Untreated	–	26.0 ± 0.2
Genistein	0.5 mg/kg × 4	25.0 ± 0.2**
	10 mg/kg × 4	25.5 ± 0.2*
Resveratrol	0.5 mg/kg × 4	25.7 ± 0.1
	10 mg/kg × 4	26.0 ± 0.1
Zearalenone	0.5 mg/kg × 4	24.8 ± 0.1**
	10 mg/kg × 4	24.4 ± 0.2**
Bisphenol A	0.5 mg/kg × 4	25.8 ± 0.2
	10 mg/kg × 4	24.8 ± 0.2**
Diethylstilbestrol	0.5 μg/kg × 4	24.5 ± 0.1**
	10 μg/kg × 4	24.1 ± 0.2**

Values represent mean ± S.E. Each group consists of 24 mice.
* $P < 0.05$, compared with untreated controls.
** $P < 0.01$, compared with untreated controls.

Table 2
Estrous cycle alteration in female offspring of mothers exposed to xenoestrogen (four daily injections, beginning on gestational day 15)

Test chemical	Dose	One cycle length	Percent of time spent in	
			Estrus	Diestrus
Untreated	–	5.2 ± 0.1	18.7 ± 0.4	24.2 ± 2.1
Genistein	0.5 mg/kg × 4	6.4 ± 0.3**	15.9 ± 0.7	31.0 ± 1.7**
	10 mg/kg × 4	7.2 ± 0.4**	14.3 ± 0.8	34.5 ± 1.8**
Resveratrol	0.5 mg/kg × 4	6.5 ± 0.2**	14.7 ± 0.9	33.7 ± 2.7**
	10 mg/kg × 4	6.1 ± 0.2**	15.1 ± 0.5	29.8 ± 1.7
Zearalenone	0.5 mg/kg × 4	7.7 ± 0.3**	29.8 ± 4.7**	29.8 ± 3.0
	10 mg/kg × 4	10.9 ± 0.5**	48.4 ± 4.0**	23.4 ± 2.2
Bisphenol A	0.5 mg/kg × 4	8.0 ± 0.4**	13.1 ± 0.6	38.9 ± 2.0**
	10 mg/kg × 4	8.2 ± 0.3**	13.9 ± 0.4	40.5 ± 1.2**
Diethylstilbestrol	0.5 μg/kg × 4	5.5 ± 0.1	17.5 ± 0.7	30.2 ± 2.1*
	10 μg/kg × 4	5.8 ± 0.1*	17.5 ± 0.9	37.3 ± 1.7**

Values represent mean ± S.E. (days).
* $P < 0.05$, compared with untreated controls.
** $P < 0.01$, compared with untreated controls.

3.4. Reproductive tract structure

Ovarian histology in mice sacrificed at 4 weeks of age revealed that, compared with untreated control mice (Fig. 2a), corpora lutea were absent in high-dose (but not low-dose) GEN-, RES- and ZEA-treated mice (Fig. 2b) (2/6, 1/6 and 5/6, respectively), and in both low- and high-dose BPA (2/6 and 3/6, respectively) and DES-treated mice (5/6 and 6/6, respectively) (Table 3). However, corpora lutea was present in GEN-, RES-, BPA- and DES-treated mice sacrificed at 8, 12 or 16 weeks of age. In contrast, corpora lutea were absent in high-dose ZEA-treated mice sacrificed at 8, 12 or 16 weeks of age (6/6, 5/6 and 2/6, respectively); i.e., the number of mice without corpora lutea decreased with advancing age. In some ZEA-treated mice without corpora lutea, we observed ovarian interstitial cell hyperplasia composed of light-staining cells arranged in tubule-like structures, and/or squamous metaplasia of uterine glands. The other tested chemicals produced no abnormalities in the uterus. Vaginal cornification was observed in mice lacking corpora lutea, but none of the tested chemicals induced vaginal epithelial abnormality.

3.5. Mammary gland

In untreated control mice at 4 weeks of age, TEBs were observed at the periphery with lateral buds, whereas alveolar differentiation was unclear (Fig. 3a; Score 1). At ≥8 weeks of age, the alveolus and lobulus appeared. At 12 and 16 weeks of age, mammary glands showed no further differentiation. At 8, 12 and 16 weeks of age, the degree of development varied somewhat from animal to animal. In some cases mammary glands were relatively poorly differentiated, with small number of alveoli within poorly developed ducts

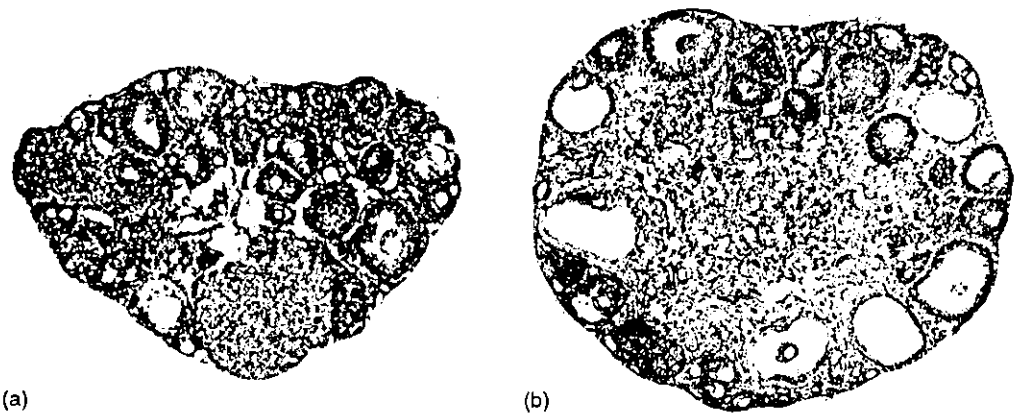

Fig. 2. Ovaries from 4-week-old CD-1 mice. (a) Mouse exposed prenatally to DMSO vehicle. Note prominent corpora lutea. (b) Mouse exposed prenatally to 10 mg/kg × 4 ZEA. Note absence of corpora lutea and conspicuous interstitial cell hyperplasia.

Table 3
Absence of corpora lutea in female mice treated with xenoestrogen prenatally

Xenoestrogens	Dose	No. of mice without corpora lutea (%)			
		4	8	12	16 (weeks)
Untreated	–	0	0	0	0
Genistein	0.5 mg/kg × 4	0	0	0	0
	10 mg/kg × 4	2 (33)	0	0	0
Resveratrol	0.5 mg/kg × 4	0	0	0	0
	10 mg/kg × 4	1 (17)	0	0	0
Zearalenone	0.5 mg/kg × 4	0	0	0	0
	10 mg/kg × 4	5 (83)	6 (100)	5 (83)	2 (33)
Bisphenol A	0.5 mg/kg × 4	2 (33)	0	0	0
	10 mg/kg × 4	3 (50)	0	0	0
Diethylstilbestrol	0.5 μg/kg × 4	5 (83)	0	0	0
	10 μg/kg × 4	6 (100)	0	0	0

(Fig. 3b; Score 2); other cases showed greater ductal and alveolar development (Fig. 3c; Score 3), and some exhibited fully developed lobulo-alveolar formation (Fig. 3d; Score 4). At 4 weeks of age, all untreated controls had Score 1 mammary glands, whereas all high- and low-dose ZEA-treated mice and 2 out of 3 high-dose BPA-treated mice with corpora lutea showed alveolar differentiation (≥Score 2) (Fig. 4a) with some alveoli showing secretory activity (Fig. 4b); however, at ≥8 weeks of age, none of the xenoestrogen-treated mice showed adverse effects on growth of mammary glands. In contrast, ZEA-treated mice lacking corpora lutea at 8, 12 and 16 weeks of age exhibited dilated beaded ducts without alveolar formation (Score 1) (Fig. 5a); these dilated ducts contained an eosinophilic substance (Fig. 5b). Scores of mammary gland differentiation after perinatal xenoestrogen exposure are summarized in Fig. 6. At 4 weeks of age,

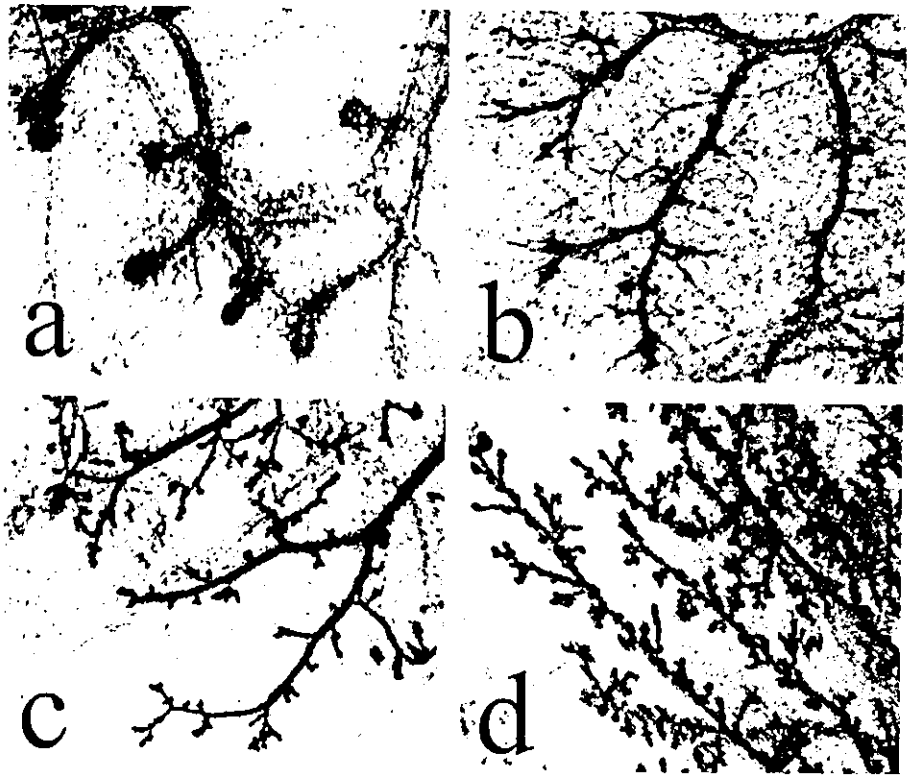

Fig. 3. Mammary glands from untreated CD-1 mice. (a) Four-week-old mouse. Note terminal end buds at the periphery with lateral bud but no alveolar differentiation (Score 1). (b) Twelve-week-old mouse. Note small number of alveoli within poorly developed duct (Score 2). (c) Twelve-week-old mouse. Note more advanced ductal and alveolar development, compared with panel (b) (Score 3). (d) Twelve-week-old mouse. Note prominent lobulo-alveolar development (Score 4).

Fig. 4. Mammary gland from a 4-week-old ZEA-treated CD-1 mouse with intact ovary. (a) Note terminal end buds at the periphery with advanced alveolar differentiation (compare with Fig. 3a). (b) Alveoli exhibit secretory activity.

Fig. 5. Mammary gland from a 16-week-old ZEA-treated CD-1 mouse without corpora lutea. (a) Note dilated beaded mammary ducts without alveolus. (b) Dilated duct contains eosinophilic substance.

accelerated mammary gland differentiation was observed in ZEA- and BPA-treated mice (with corpora lutea). At 8–16 weeks of age, growth retardation was observed in high-dose ZEA-treated mice (without corpora lutea).

4. Discussion

In the present study, in utero exposure to xenoestrogens (phytoestrogens, mycoestrogens and industrial chemicals) at doses equivalent to typical human exposure and at a 20-fold higher dose produced various degrees of alteration in reproductive tract and mammary gland in female CD-1 mice. In previous studies, perinatal treatment with DES or GEN did not alter body weight [6]. ZEA and BPA have been found to increase fetal body weight, with the effect of BPA continuing into adulthood [14,15]. In the present study, at 16 weeks of age, body weight was significantly increased in all treatment groups other than low-dose DES.

Neonatal administration of estrogen or androgen causes earlier vaginal opening in rats [16]. Prenatal exposure to 2 μg/kg DES or 20 μg/kg BPA [17], and neonatal exposure to 0.4 mg/kg GEN or 0.04 mg/kg ZEA, causes earlier vaginal opening in mice [18]. In rats, prepubertal exposure to 10 mg/kg ZEA or 100 mg/kg RES causes earlier vaginal opening [19,20]. The present finding that all chemicals